Molecular mechanics of nucleic acid sequence amplification

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Protocols for in vitro amplification of nucleic acids are proliferating and there are now several methods that will contribute both to genetic research and to the diagnosis of a wide range of diseases. Here, I present the working principles of some of these molecular machines for amplifying DNA or RNA and discuss the lines along which new methods of amplifying nucleic acids may be developed.

The ability to replicate genetic material while preserving its information content is a fundamental property of a genetic system. A rudimentary understanding of the underlying principles has long been useful in the breeding of animals, plants and microorganisms that have valuable properties. With recombinant DNA technology, a gene of interest can be excised from its natural context and replicated in other organisms. Reactions in which nucleic acids are amplified *in vitro* can be viewed as a third stage in this development, permitting replication of specific DNA or RNA sequences without the use of carrier organisms. A basic principle that is common to all *in vitro* amplification techniques is that base pairing of specific nucleic acid probes initiates the enzymatic accumulation of more copies of specific nucleic acid molecules.

Methods for replicating nucleic acid sequences are a focus of interest in studies of the origin of life[1]. Early studies of the autocatalytic replication of RNA sequences using the Q_β phage also provided insights into the diagnostic potential of amplification reactions[2]. Another method of nucleic acid amplification, the polymerase chain reaction (PCR), was proposed more than 20 years ago[3], but it was not until 1985 that the technique was presented in a practical form[4]. Today, PCR is by far the most important technique used to amplify nucleic acid sequences *in vitro*: thousands of worker-years have been invested in refining and modifying this technique. However, several other techniques have been developed that either amplify a nucleic acid sequence present in a sample or that use nucleic acids to amplify the signal from a detection reaction. As discussed here, these techniques differ in some fundamental respects, and it may be expected that yet more methods for nucleic acid replication, based on similar or novel mechanisms, will be developed. (For a comparison of amplification techniques, see also Refs 5–7.)

An assortment of molecular amplifiers

The polymerase chain reaction

In PCR, both strands of a target DNA sequence are replicated by enzymatic DNA synthesis, initiated from two oligonucleotide primers. The temperature of the reaction is varied cyclically to allow denaturation of the DNA template, followed by hybridization of the primers to the target sequence, and DNA synthesis. In each cycle the number of copies of the target segment is approximately doubled, resulting in exponential amplification[4,8,9] (Fig. 1; reviewed in Refs 10, 11).

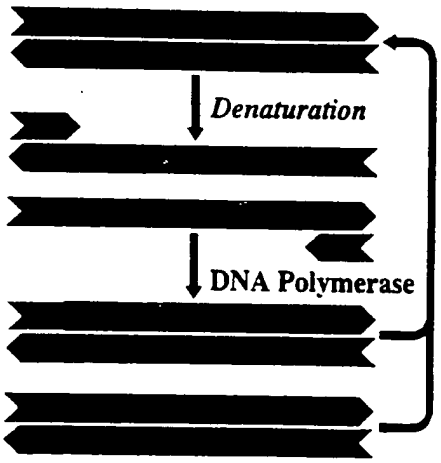

FIG 1

The polymerase chain reaction (PCR). The target DNA segment is denatured and two oligonucleotide primers hybridize to the two strands. DNA polymerase synthesizes new strands from the primers, doubling the number of copies of the target sequence. Synthetic oligodeoxynucleotides are shown in light blue and other DNA strands in dark blue. The 5'→3' orientation of nucleic acid molecules is indicated by broad arrowheads at their 3' ends. The thinner black arrows indicate the flow of events.

Exponential probe ligation

The ligation amplification reaction[12] (LAR), or ligase chain reaction[13,14] (LCR), involves a cyclical accumulation of the ligation products of two pairs of complementary oligonucleotides. The sequence of the oligonucleotides is identical to the target sequence, fragmented as two double-stranded segments. The amplification reaction is triggered by the presence of the proper target sequence: this permits the ligation of pairs of oligonucleotides that hybridize in juxtaposition on each strand of the target segment. These ligation products are recruited as templates for the ligation of more primers in subsequent cycles, a process which is controlled by varying the incubation temperature (Fig. 2). Special measures can reduce the risk that the probes ligate independently of a target sequence through ligation between single-stranded sequences or blunt ends[15].

LCR does not amplify copies of any genomic sequence other than that represented by the oligonucleotides added, since the product is entirely composed of ligated copies of the input probes. The process can thus be viewed as a detection reaction, followed by a signal amplification procedure.

Segev[16] has described an amplification technique (P&LCR) that combines features of the polymerase and the ligase chain reactions. In this procedure, the combined action of a polymerase and a ligase is required to close the gap between pairs of oligonucleotides that hybridize some distance apart on each strand of the target sequence. The nucleic acid strands thus formed then serve as templates for polymerization and ligation events, which can be controlled by temperature cycling.

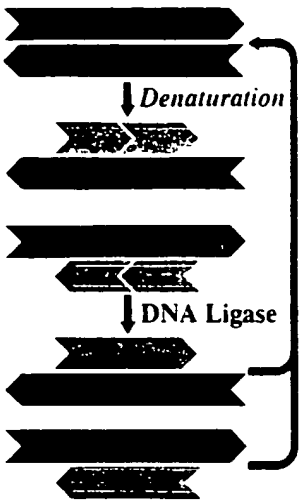

FIG 2

The ligase chain reaction (LCR). Two pairs of oligonucleotides hybridize in immediately adjacent positions to each strand of a denatured target sequence. After ligation and denaturation, the ligated oligonucleotides are recruited as templates for new cycles of hybridization and ligation. Synthetic oligodeoxynucleotides are shown in light blue and other DNA strands in dark blue.

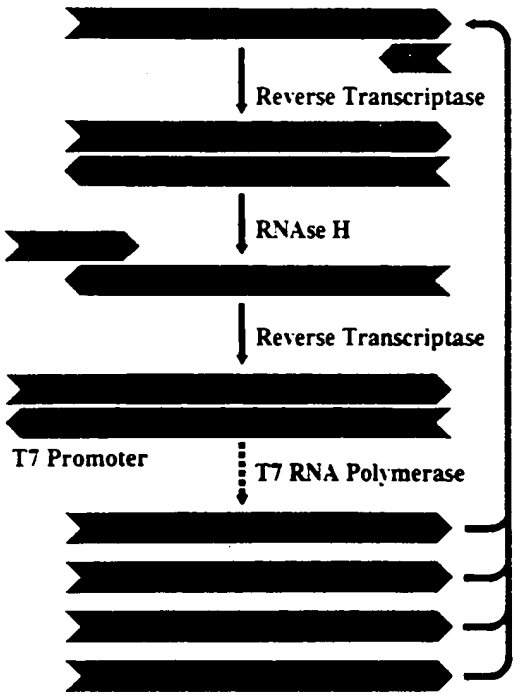

FIG 3

The self-sustained synthetic reaction (3SR) or nucleic acid sequence based amplification (NASBA). A primer complementary to the 3' end of a target RNA sequence initiates DNA synthesis by reverse transcriptase. The RNA template is removed by the action of RNase H, permitting another primer to hybridize to the newly synthesized cDNA molecule. This primer includes at its 5' end the recognition sequence for a viral transcriptional polymerase. After the DNA copy of the target molecule has been rendered double-stranded by reverse transcriptase, the RNA polymerase synthesizes numerous RNA molecules, similar or identical to the original target sequence, thus completing the cycle. Synthetic oligodeoxynucleotides are shown in light blue, other DNA strands in dark blue and RNA strands in red.

Cyclical transcription and reverse transcription

While replication or ligation reactions that are primed from a DNA template yield one doubling of the number of target sequences during each cycle, a transcription reaction in which the DNA segment is preceded by an appropriate promoter can yield 10–1000 RNA copies of the desired DNA sequence[17]. Coupling the transcription reaction to reverse transcription of the RNA molecules results in a cyclical accumulation of copies of the target sequence.

In two similar techniques, self-sustained sequence replication[18,19] (3SR) and nucleic acid sequence-based amplification[20] (NASBA), an amplification cycle begins when the enzyme reverse transcriptase synthesizes a cDNA strand from an RNA template, using an oligonucleotide primer that is complementary to part of the RNA sequence. Next, RNase H specifically degrades all copied RNA molecules. This allows a second primer to hybridize to the cDNA molecules and initiate synthesis of a complementary strand. The second primer includes a promoter sequence that is rendered double-stranded in the copying process. Once double-stranded, the promoter permits transcription of the downstream sequence by a viral RNA polymerase. Up to 100 copies of the original RNA sequence are generated from each of these molecules, and each of these in turn serves as a template in subsequent cycles of amplification (Fig. 3).

In the 3SR and NASBA reactions there is spontaneous cycling between the different enzymatic phases at a fixed temperature, resulting in exponential amplification of the target sequence. DNA templates may also be amplified, by varying the reaction temperature for a few cycles to generate an RNA copy. The reaction can then proceed spontaneously. In an earlier amplification procedure that is also based on transcription, the transcription-based amplification system (TAS), reverse-transcribed RNA molecules are separated from cDNA molecules by heat denaturation rather than by enzyme digestion[21].

Strand displacement and amplification

One of the fundamental problems in amplification reactions is that of dissociating the nucleic acid duplexes that are formed. In a recent addition to the small group of isothermal amplification techniques, strand displacement amplification (SDA), this problem is solved by the displacement of the complementary DNA strand from a target sequence during the synthesis of a new strand[22,23] (Fig. 4). A DNA sample is digested with restriction enzymes and subjected to an initial denaturation. A specific primer is then hybridized to the end of one strand of the fragment of interest. DNA polymerase extends the primer and also extends the template molecule by copying the 5' end of the primer. During this synthesis reaction, modified dATP nucleotides are incorporated. Since the incorporated A residues have a sulphur in the alpha position, the primer, but not its DNA copy, is cleaved by a restriction enzyme that recognizes a site at the 5' end of the primer. DNA synthesis is initiated at this nick, but the DNA polymerase used lacks the 5' exonucleolytic activity required for nick translation. Instead, the downstream fragment is released by strand displacement

during DNA synthesis. Successive cycles of hemi-restriction and strand displacement follow. The result is an exponential amplification of the target DNA, since displaced strands are recruited as templates for an analogous reaction on the other strand of the target sequence.

In this procedure, the DNA sample is restriction digested to permit the addition of end sequences by copying the 5' end of a primer. Alternatively, a single strand that has been synthesized from a primer with the required 5' extension can be released from a template strand by strand displacement from another, upstream primer. The released strand can then be replicated without any denaturation step[23].

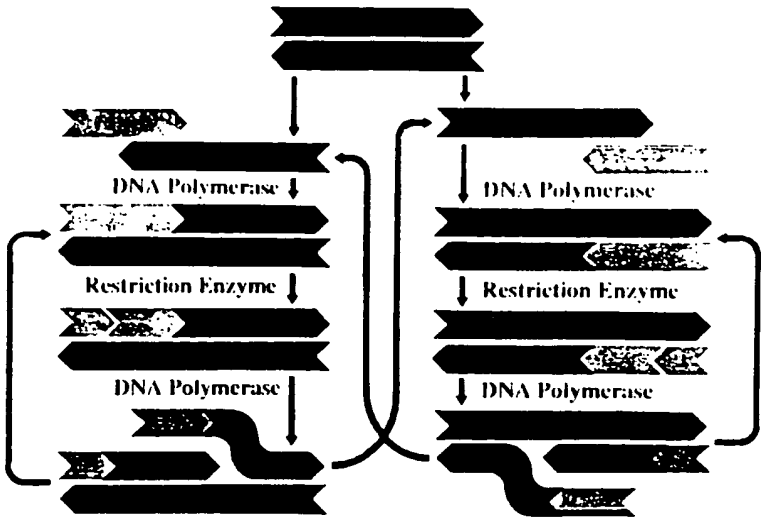

FIG 4

Strand displacement amplification (SDA). An oligonucleotide primer hybridizes to one strand of a denatured target sequence. The molecule, including the noncomplementary 5' end of the primer, is made double-stranded by the action of a DNA polymerase. The 5' end of the primer contains the recognition sequence for a restriction endonuclease. However, since modified nucleotides are incorporated during DNA synthesis, only the primer is susceptible to cleavage by the restriction enzyme. DNA synthesis is initiated at the nicked site but the DNA polymerase used is incapable of nick translation. Instead, the DNA strand downstream of the nick is displaced. The newly synthesized strand is also nicked, leading to successive cycles of strand displacement. The displaced strands are recruited as templates for an analogous reaction on the other strand of the target sequence, with extension from another primer, followed by hemi-restriction and strand displacement. Synthetic oligodeoxynucleotides are shown in light blue and other DNA strands in dark blue.

Probe amplification by Q_β replicase

An RNA-dependent RNA polymerase, derived from the bacteriophage Q_β, can be used for exponential amplification of RNA probes. The probes are designed to include sequence elements required for replication by Q_β replicase, along with a segment that is complementary to the sequence to be detected. Assays are set up so that probes will be captured in a reaction only if they hybridize to the appropriate target sequence in the added sample. Probe molecules that remain after washing are exposed to the Q_β replicase, resulting in a 10^8-fold amplification of these molecules during a 15 minute incubation at a fixed temperature[24] (Fig. 5).

In order to take advantage of the potential of Q_β replicase to detect target sequences that are present at low copy numbers, it is important to minimize nonspecific binding of probes. This requirement has been met by incorporating into the detection scheme an automated succession of steps for probe capture and release[25,26]. By this means, probe molecules that are bound nonspecifically to reaction vessels or solid supports are removed and do not contribute to background in the assay. A number of other strategies have been devised to ensure that replication templates are available in an assay only if the specific target sequence was added to the reaction[27,28].

Accumulation of the products of probe digestion

In most amplification procedures the reaction products accumulate exponentially. The cycling probe technique is an exception in that it is based on the degradation of probes at a constant rate, thus limiting the speed and level of amplification that can be attained. A synthetic oligonucleotide probe is designed to include a segment of ribonucleotide residues, surrounded by deoxynucleotides. Upon hybridization to the target sequence, the ribonucleotide segment becomes susceptible to digestion by RNase H. The resultant probe fragments spontaneously dissociate from the intact target sequence, leaving the target free to sequentially hybridize to, and trigger the digestion of, more probe molecules. Probe fragments accumulate at a constant rate during incubation and this process can be monitored to reveal the presence of the target sequence[29] (Fig. 6). Another linear amplification method has been described in which lambda exonuclease is used to specifically degrade hybridized, but not free, DNA probes[30].

Distinctive features of amplification protocols

The amplification techniques described differ in a number of respects, and these influence their suitability for use in, for example, diagnostic assays (Table 1). The techniques can be broadly divided between those which increase the copy number of a nucleic acid sequence found in the sample, and those which merely serve to amplify the signal from a nucleic acid detection reaction. The former techniques lend themselves to the investigation of the nucleotide sequence of a DNA or RNA segment (for instance, to search for mutations). Use of the latter techniques is restricted to identifying or quantifying specific sequences, or distinguishing between known sequence variants.

As discussed below, the specificity with which a unique target sequence may be identified is influenced by the number of probes required in the various techniques. The increasing number of thermostable enzymes that are available in the toolbox for amplification reactions also contributes to increased specificity[7]. Techniques that are performed at a fixed temperature have an obvious advantage in routine diagnostic tests, since temperature cycling equipment is not required.

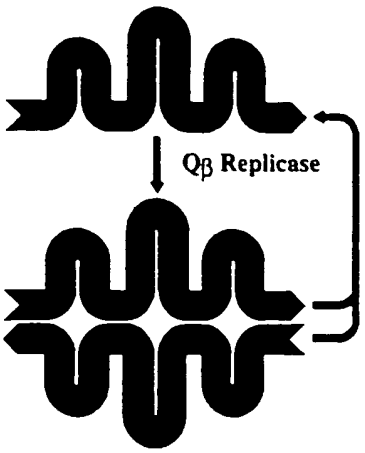

FIG 5

Amplification by Q_β replicase. An RNA probe that contains an internal segment complementary to the target sequence is recognized by Q_β replicase, which synthesizes a complementary copy. Both the copy and the original molecule then serve as templates for further cycles of replication. The prominent secondary structure of the probe prevents the replicated molecules from collapsing to form a linear duplex. RNA strands are shown in red.

Mechanics of amplification

The various techniques represent different solutions to a set of problems that are inherent to nucleic acid amplification. Reactions are initiated by hybridization between probe and target sequences. Techniques in which hybridization to two or more independent target sequences is required to initiate amplification show the greatest specificity in detecting unique target sequences from complex DNA or RNA samples. Specificity is further increased by use of a probe that also serves as a primer for DNA synthesis, because the 3' end of the probe must pair precisely with a target sequence in order for the primer to be recognized by the polymerase[31]. Likewise, in LCR, the region where two hybridizing oligonucleotides meet must be correctly matched for the ligase to join them[13,32,33]. Since to prime amplification in LCR, two probes must hybridize in immediately adjacent positions this further reduces the risk that the process will be initiated by a fortuitous match between the probes and irrelevant sequences in a DNA sample. Stringent requirements for the initiation of amplification are crucial in the development of standardized diagnostic protocols.

Once initiated, most of the amplification methods, whether based on enzymatic synthesis of DNA and/or RNA, on ligation or on nucleolysis, continue to depend on the same type of probe hybridization. Q_β amplification is an exception in that ongoing replication depends only on protein–RNA interactions between the replicase and its template.

The techniques described here exemplify five different means by which duplexed nucleic acid molecules can be dissociated or disrupted to allow amplification to proceed. In one group of amplification techniques (PCR, LCR, P&LCR and TAS) the reactions are periodically heated to denature base-paired molecules and to initiate new replication cycles. As an alternative, these reactions could perhaps be cyclically denatured at a fixed temperature by periodically subjecting the samples to an electric field[34]. In 3SR and NASBA, RNA molecules hybridized to DNA strands are removed by the enzyme RNase H, or by the RNase H activity inherent to reverse transcriptase[18], obviating the need for temperature cycling. In the SDA technique, base-paired DNA strands are separated by strand displacement as a new strand is being synthesized in place of the old one. Efficient templates for Qβ replication typically show prominent secondary structure, which prevents the complementary strands from collapsing to a straight double helix while a new strand is being synthesized as the helix is 'unzipped'. Lastly, in the cycling probe approach, the probe dissociates from the target sequence after being cleaved by an enzyme that recognizes paired probes, as the result of unstable hybridization of the remaining fragments.

Clearly, for each of the principal steps in the amplification cascade – initiation, progression and resolution – there are alternative mechanisms to be explored.

TABLE 1. Properties of *in vitro* amplification techniques[a]

	PCR	LCR	P&LCR	3SR/NASBA	TAS	SDA	Q_β	Cycling probe
Amplifies a target sequence	+		+	+	+	+		
Dependent on recognition of two independent sequences	+	+	+	+	+	+		
Performed at high temperature	+	+	+					
Operates at a fixed temperature				+		+	+	+
Results in exponential amplification	+	+	+	+	+	+	+	

[a]For full names of techniques and detailed discussion, see text.

Outlook

More molecular machines

The rights to the diagnostic applications of PCR were sold for 300 million dollars. At this price, it seems likely that the search for new amplification methods will continue. New amplification methods could be developed along several lines: new protocols may involve mixing and matching elements of existing techniques, perhaps with a new approach to one of the problems encountered, such as dissociation of base paired probe–target hybrids. Another avenue towards novel amplification schemes would be to develop *in vitro* analogues of simple viral replication systems, in the same way as the Q_β amplification technique builds on the replication of bacteriophage Q_β and the 3SR and NASBA techniques resemble retroviral replication. It is possible to replicate the linear duplex DNA of bacteriophage p29 *in vitro*[35], and this technique might permit *in vitro* amplification of DNA segments as large as 100 kb[36,37]. Lastly, if ongoing work to develop enzyme-independent chemical replication systems is successful, it could one day be harnessed to amplify coding molecules[1,38,39].

For some types of genetic analysis, it is possible to avoid the need for amplification reactions by use of a combination of specific nucleic acid detection reactions and improved visualization techniques. Removing the requirement for amplification has the benefit of reducing the risk of contamination and brings with it the potential for rapid analysis of very large sets of sequences in parallel, while preserving spatial information for *in situ* analysis. Indeed, in the microscopic format of fluorescent *in situ* hybridization to chromosomes, detection of single target molecules is already an established procedure[40,41]. Nonetheless, techniques for *in vitro* amplification of nucleic acids will remain crucially important in many areas of molecular genetics.

Applications of in vitro amplification

PCR has lent credence to the aims of the Human Genome Project, helping to solve some of the logistic problems involved, including that of making genetic markers generally available[42]. In the search for nucleic acids with unique properties, several of the methods for *in vitro* amplification have been used to exploit the potential of replicating systems to evolve under selection. In this manner very complex mutations can be created and rapidly screened[43–45]. PCR using primers derived from strongly conserved sequences has also proven useful in the study of gene evolution. The etiologic agent of the intestinal affliction Whipple's disease was recently identified and assigned a taxonomic identity solely on the basis of a 1.3 kb ribosomal sequence amplified from the lesions[46].

One of the most important outcomes of the development of *in vitro* amplification techniques is that the prospect of DNA diagnostics now seems practical. It is hoped that homogeneous assays (assays that do not require washes before the outcome of the amplification is measured) can be developed. Such reactions could be sealed before amplification is initiated and a successful amplification identified through the accumulation of intercalating fluorescent dyes in the DNA or RNA products of the reaction[47] or via the cleavage of a probe sequence, digested by an advancing polymerase on an amplification template[48]. Homogeneous detection methods will be less subject to contamination from previously amplified reactions, an important concern particularly for routine applications. Simplified and robust amplification techniques may indeed become literally a household item that could permit molecular analysis in the home of the cause of a sore throat or a runny nose, and will certainly help diagnose parasitic infestations in the Third World.

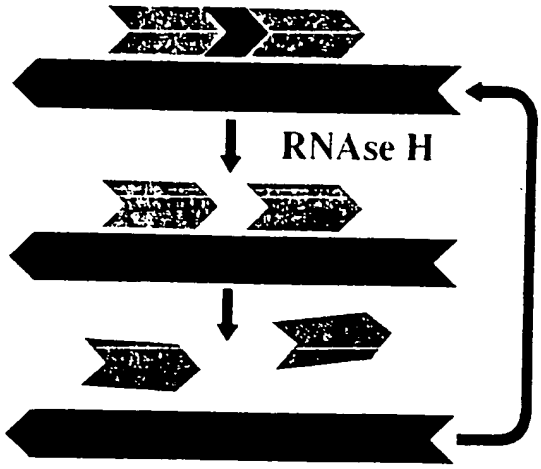

FIG 6

The cycling probe reaction. A segment of ribonucleotide residues in the middle of an oligonucleotide probe is cleaved by the enzyme RNase H after the probe has hybridized to its target sequence. The remaining fragments of probe dissociate from the target sequence, allowing another probe molecule to hybridize to the target. This probe is, in turn, cleaved. Synthetic oligodeoxynucleotides are shown in light blue, other DNA strands in dark blue and RNA strands in red.

In conclusion, the machines for *in vitro* replication can be expected to continue to evolve, both copying and increasing available genetic information. These machines are firmly establishing themselves as particularly successful examples of those autocatalytically replicating mental concepts that Richard Dawkins identified as 'memes'[49].

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